

*Application
for
United States Letters Patent*

To all whom it may concern:

Be it known that

Gabriela Chiosis, Ivo G. Boneca, and W. Clark Still

have invented certain new and useful improvements in

**METHOD FOR RE-SENSITIZING VANCOMYCIN RESISTANT BACTERIA WHICH SELECTIVELY
CLEAVE A CELL WALL PEPTIDE**

of which the following is a full, clear and exact description.

**METHOD FOR RE-SENSITIZING VANCOMYCIN RESISTANT BACTERIA
USING AGENTS WHICH SELECTIVELY CLEAVE A CELL WALL PEPTIDE**

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The invention disclosed herein was made with Government support under NIH Grant No. 5-R01-HL-25634-18 from the National Institutes of Health. Accordingly, the U.S. Government has certain rights in this invention.

Throughout this application, various references are identified by citations or a number in parenthesis, in which case their full citations appear on the pages following the Detailed Description immediately preceding the claims. Disclosure of these references in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

20

Field of the Invention

The present invention relates to a method for re-sensitizing vancomycin resistant Gram-positive bacteria in which resistance results from the conversion of an amide bond to an ester bond in the cell wall peptide precursors of the bacteria which comprises using an antibacterial amount of vancomycin or a homolog of vancomycin and an amount of an agent effective to selectively cleave the ester bond so as to thereby re-sensitize vancomycin resistant bacteria.

35

Background of The Invention

1 The introduction of the first antimicrobial agents allowed
5 physicians and patients to manage infectious diseases
effectively. Unfortunately, even before penicillin was
introduced commercially, researchers had identified the
first resistant *Staphylococcus aureus* (1). Since then,
many new antimicrobials have been developed to combat the
10 emergence of resistance. These advances increased the
confidence that infectious diseases are non-fatal and
still manageable. Nevertheless, with the medical
progresses that allow people to live longer and the boost
of various debilitating immune conditions (AIDS, cancer,
15 organ transplants), a new human population emerged as
being at great risk of infectious diseases caused in
majority by two common nosocomial pathogens,
Staphylococcus and *Enterococcus* species (2). The incidence
of staphylococcal strains resistant to virtually all the
20 antimicrobial agents except vancomycin has increased
drastically during the last decade. Additionally,
enterococcal infections are becoming increasingly
resistant even to vancomycin, raising the alarming
possibility that resistance genes will eventually be
25 transmitted to staphylococci (3-5). One of the greatest
concerns with vancomycin resistant enterococci (VRE) is
that the resistance to vancomycin will be picked up by
Staphylococcus aureus through genetic recombination. Many
forms of *S. aureus* have already become resistant to
30 methicillin and can now only be treated with vancomycin.
There is significant concern that if *S. aureus* also
becomes resistant to vancomycin, the health care

profession will be left without treatment for these types of infections. Increasing resistance among several types of Gram-positive bacteria associated with common and potentially life-threatening infections complicate the treatment of serious infections and has been linked to extended hospitalizations, higher medical costs and high mortality rates.

Like the family of β -lactam antibiotics, vancomycin acts on peptidoglycan metabolism. The peptidoglycan is essential for bacterial survival because of its function as the exoskeleton that prevents cell rupture due to internal pressure. By binding to the D-Ala-D-Ala moiety of the bacterial cell wall precursors, vancomycin interferes with the growth of the peptidoglycan (6). In the resistant strains with *vanA* or *vanB* phenotype however, some of the D-Ala-D-Ala moiety of the cell wall precursors is substituted by analogous D-Ala-D-Lac ones (7-9). Only a small percentage of the enterococcus peptidoglycan layer is needed to be structurally altered from D-Ala-D-Ala to D-Ala-D-Lac to cause an increase in the vancomycin MIC (10% of the altered peptidoglycan increases the MIC of vancomycin from 2 to 32 ug/ml).

Resistant bacteria carry a transportable element encoding nine genes that contribute to the resistance phenotype (10). These gene products include VanS, a transmembrane protein that senses directly or indirectly the presence of vancomycin. Once autophosphorylated, VanS transmits a signal to a response regulatory protein VanR that activates transcription of the other resistance genes (11). VanA is involved in the synthesis of the

depsipeptide D-Ala-D-Lac while VanH converts pyruvate into D-lactate. This pathway is essential for the resistance phenotype (12,13). VanX is a Zn^{2+} dependent peptidase that selectively cleaves D-Ala-D-Ala leading to an accumulation
5 of the depsipeptide, and thus, of precursors with altered D-Ala-D-Lac termini (14,15). VanY is a membrane bound D-D-carboxypeptidase that hydrolyses the normal cell wall precursor lipid-intermediates, further increasing the pool of precursors with altered termini (16). However, the
10 formation of D-Ala-D-Ala continues in the cell due to the activity of the native enterococcal D-Ala-D-Ala ligase. Because vancomycin binds to D-Ala-D-Ala substrates, a mechanism is required to prevent D-Ala-D-Ala from being incorporated into the cell wall. VanX and vanY perform
15 this function. As a result of the incorporation of D-Ala-D-Lac by vancomycin resistant enterococcus (VRE), the affinity of vancomycin for the peptidoglycan layer diminishes over 1000-fold, leading to antibiotic resistance. VanA strain is the most common phenotype of
20 VRE and is described by inducible, high-level resistance that is associated with the *van* genes that lead to D-Ala-D-Lac altered termini.

In order to bypass resistance, vancomycin has been
25 modified to enhance its binding to D-Ala-D-Lac and inhibitors of the D-Ala-D-Lac biosynthetic pathway have been sought (17-19). Here we propose another approach- the selective and catalytic cleavage of the D-Ala-D-Lac depsipeptide by small molecules. By reducing the
30 concentration of precursors with altered termini one would expect to re-sensitize the bacteria to vancomycin. A small molecule that performs such task could be used in concert

with vancomycin (or vancomycin derivatives with higher affinity) in the treatment of *vanA* resistant strains.

[illegible]

Summary of the Invention

One of the most challenging situations for the immuno-
compromised patients is the development of vancomycin-
5 resistant enterococci (VRE), an increasingly frequent
cause of hospital-acquired infections in the United
States. These organisms are resistant to virtually all
currently available antibiotics including vancomycin,
considered the agent of last resort for Gram-positive
10 infections. VanA strain is the most common phenotype of
VRE and is described by inducible, high-level resistance
that is associated with the *van* genes that lead to D-Ala-
D-Lac cell wall altered termini. Here we describe the
development of small molecules that catalytically and
15 selectively cleave the altered termini of the bacteria
cell wall so as to disable the antibiotic-resistance
mechanism in these pathogens. The molecules re-sensitize
bacteria to the drug and could be used in concert with
vancomycin in the treatment of VRE.

20 The invention provides a method of treating a subject
afflicted with an infection caused by glycopeptide
antibiotic resistant Gram-positive bacteria, such as
vancomycin resistant Gram-positive bacteria, in which
25 resistance results from the conversion of an amide bond to
an ester bond in the cell wall peptide precursors of the
bacteria which comprises administering to the subject an
antibacterial amount of glycopeptide antibiotic, such as
vancomycin or a homolog of vancomycin, and an amount of an
30 agent effective to selectively cleave the ester bond so as
to thereby treat the subject.

The invention also provides a method of killing glycopeptide antibiotic resistant Gram-positive bacteria, such as vancomycin resistant Van A, Van B, Van D, or Van G
5 Gram-positive bacteria which comprises contacting the bacteria with an agent that selectively cleaves D-Ala-D-Lac cell wall depsipeptides in the bacteria in an amount effective to cleave such depsipeptides and an antibacterial amount of glycopeptide antibiotic, such as
10 vancomycin or a homolog of vancomycin, so as to thereby kill the bacteria.

The invention further provides a method for determining whether a test compound selectively cleaves an ester bond
15 present between two amino acid-like moieties in a depsipeptide which comprises contacting a compound comprising the structure X-Y, wherein each of X and Y are amino acid-like moieties and - is an ester bond with the test compound and determining whether the test compound
20 cleaves the ester bond.

In another aspect, the invention provides a method of treating a subject afflicted with an infection caused by glycopeptide antibiotic resistant Gram-positive bacteria
25 in which resistance results from the conversion of an amide bond to an ester bond in the cell wall peptide precursors of the bacteria which comprises administering to the subject an antibacterial amount of a glycopeptide antibiotic and an amount of an agent effective to
30 selectively cleave said ester bond so as to thereby treat the subject.

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Brief Description of the Drawings

Figure 1. Combinatorial libraries selected to screen for cleavage of D-Ala-D-Lac.

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Figure 2. Computer generated model of the complex of BnNHL-Lys-D-Pro-L-Ser dimethylurea **4a** with PhNH-D-Ala-D-Lac. Calculated hydrogen bonds are depicted by dashed lines.

10

Figure 3. Schematic representation of the small molecules assayed with the depsipeptide and the intermediate resulted by the nucleophilic attack of the serine.

15 **Figure 4.** Representative kinetic data for the hydrolysis of depsipeptide derivative **5** by 20mM phosphate buffer (pH = 7.0) (triangles), BnNHL-Lys-D-Pro-L-Ser dimethylurea **4a** (filled circle), control sequence **6** (diamond), control sequence **7** (filled triangle), control sequence **8** (box),
20 control sequences **6+8** (circle). Assays were performed with depsipeptide derivative **5** at 0.5mM, while peptides were run at 12mM. The graph is the average of five separate runs.

25 **Figure 5.** Schematic representation of the S-prolinol derivatives.

Figure 6. Representative kinetic data for the hydrolysis of depsipeptide derivative **5** by 20mM phosphate buffer
30 pH=7.0 (circles), **SProDAla** (squares), **SProUC4** (diamonds), **SProC1** (asterisks), **SProC2** (crosses), **SProC3** (triangles),

SProC4 (filled circles), **SProC5** (filled squares), **SProC6** (filled diamonds).

Figure 7. Structure of the complex of PhD-Ala-D-Lac with **SProC5** as calculated by molecular modeling. Hydrogen bonds are depicted by dashed lines.

Figures 8A-8D. Illustration of the specific synergistic effect of **SProC5** against the *vanA* strain EF228. **A)** Strain EF228 was grown in the presence of increasing concentration of vancomycin with (open squares) or without (solid squares) the small molecule **SProC5** (50 mM). Bactericidal activity was estimated by determining the number of colony forming units per ml (CFU/ml) that survived the combined treatment of vancomycin and **SProC5** (solid bars). **B)** Two structurally related molecules **SProC2** (solid symbols) and **SProC5** (open symbols) were compared for their relative synergy with vancomycin. Their activities were concentration dependent (0, 5 10 and 50 mM, represented by square) with **SProC5** being the most efficient molecule mirroring the kinetic assays (see Figure 6). **C)** **SProC5** specificity together with vancomycin against strain EF228 was tested by comparing to its enantiomer **RProC5** or its 5 carbon unit **C5**. Results represent the average of four independent experiments at the fixed concentration of 100 mM. **D)** Strain JH2-2 was used as a representative of vancomycin susceptible strains that do not synthesize altered D-Ala-D-Lac terminating cell wall precursors.

Figures 9A-9B. (A) Picture of a combinatorial library after screening against substrate **1** and removal of the solution (B) Picture of the same library after several DMF washes.

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Figure 10. Photograph of library SSY before picking the red-label carrying active beads. Assay carried out at 0.85 mM (**1**) in DMF. One red bead selected for analysis is pointed out in the upper-left corner of the image.

10

Figure 11. Methodology utilized for the synthesis of dye-labeled depsipeptide (**1**)(a). 2-(Trimethylsilyl)ethanol, Ti(*i*-PrO)₄, THF; (b). BocD-AlaCOF, DIEA, DMAP, DCM; (c). TFA:DCM=4:1; (d). A, DIEA, DMAP, DCM; (e). TBAF; (f). 15 Methyl 4-hydroxybenzoate, DEAD, PPh₃, toluene:DCM=5:1; (g). LiOH; (h). Cyanuric fluoride, pyridine, DCM.

Figure 12. General procedure for the synthesis of compounds ProCn.

20 **Figure 13.** Synthesis of SProUC4. (a) BocON, TEA; (b) phosgene, **B**, pyridine; (c) NaBH₄; (d) TFA.

Figure 14. Representative kinetic data for the hydrolysis of depsipeptide derivative **5** by 20 mM phosphate buffer (pH 25 7.0) (triangles), BnNHL-Lys-D-Pro-L-Ser dimethylurea **4a** (filled circle), control sequence **6** (diamond), control sequence **7** (filled triangle), control sequence **8** (box), control sequences **6+8** (circle). Assays were performed with depsipeptide derivative **5** at 0.5 mM, while peptides were 30 run at 12 mM. The graph is the average of five separate runs.

5

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Figure 17. Representation of the ^1H NMR spectra **(A)** of compound **9**, **(B)** of BnNHL-Lys-D-Pro-L-Ser **4a** and **(C)** of THF-5% water assay mixture.

Detailed Description of the Invention

This invention provides a method of treating a subject afflicted with an infection caused by glycopeptide
5 antibiotic resistant Gram-positive bacteria, such as vancomycin resistant Gram-positive bacteria, in which resistance results from the conversion of an amide bond to an ester bond in the cell wall peptide precursors of the bacteria which comprises administering to the subject an
10 antibacterial amount of vancomycin or a homolog of vancomycin and an amount of an agent effective to selectively cleave the ester bond so as to thereby treat the subject.

15 In one embodiment of the invention, the subject is a human being.

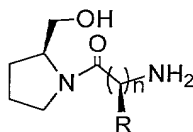
In general, the agent is an activated nucleophile and is further characterized by the presence within the agent of
20 an electrophile and chirality complementary to a bacterial cell wall depsipeptide.

In one embodiment of the invention, the agent is represented by the formula S-Pro-C_n.

25

In another embodiment of the invention, the agent has the structure:

30



wherein n is an integer from 1 to 6 inclusive and R is

hydrogen or a C₁ to C₆ straight chain or branched alkyl group.

Preferably, the agent catalytically cleaves the ester bond
5 in the cell wall peptide precursors, for example, the ester bond in the structure D-Ala-D-Lac.

The agent may be administered prior to administering a glycopeptide antibiotic, such as vancomycin or the homolog
10 of vancomycin. Specifically, the agent is administered a sufficient period of time prior to administering vancomycin or the homolog of vancomycin to permit cleavage of the ester bond in the cell wall peptide precursors to be effected.

15 Alternatively, the agent and glycopeptide antibiotic, such as vancomycin or the homolog of vancomycin are administered simultaneously, for example, the agent may be covalently attached to vancomycin or the homolog of
20 vancomycin.

In the practice of the invention, the bacteria are typically Van A, Van B, Van D or Van G Gram positive
bacteria.

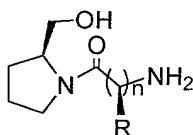
25 In another preferred embodiment of the invention, the bacteria may be Staphylococcus bacteria, S. aureus bacteria, Enterococcus bacteria, Streptococcus bacteria, Leuconostoc bacteria, Pediococcus bacteria, Lactobacillus
30 bacteria, and Erysipelothrix bacteria.

10

25

In one embodiment of the invention, the invention provides
30 a method of killing glycopeptide antibiotic resistant
Gram-positive bacteria, such as vancomycin resistant Van
A, Van B, Van D, or Van G Gram-positive bacteria which

comprises contacting the bacteria with an agent that selectively cleaves D-Ala-D-Lac cell wall depsipeptides in the bacteria in an amount effective to cleave such depsipeptides and an antibacterial amount of glycopeptide antibiotic, such as vancomycin or a homolog of vancomycin, so as to thereby kill the bacteria, wherein the agent has the structure:



wherein n is an integer from 1 to 6 inclusive and R is hydrogen or a C₁ to C₆ straight chain or branched alkyl group.

The invention provides a method of killing glycopeptide antibiotic resistant Gram-positive bacteria, such as vancomycin resistant Van A, Van B, Van D, or Van G Gram-positive bacteria which comprises contacting the bacteria with an agent that selectively cleaves D-Ala-D-Lac cell wall depsipeptides in the bacteria in an amount effective to cleave such depsipeptides and an antibacterial amount of glycopeptide antibiotic, such as vancomycin or a homolog of vancomycin, so as to thereby kill the bacteria, where the agent preferably, catalytically cleaves the ester bond in the D-Ala-D-Lac depsipeptide.

In the practice of the invention, the agent is administered prior to administering the glycopeptide antibiotic, such as vancomycin or the homolog of vancomycin, desirably a sufficient period of time prior to

administering vancomycin or the homolog of vancomycin to permit cleavage of the ester bond to be effected in the D-Ala-D-Lac depsipeptide.

5 Alternatively, the agent and the glycopeptide antibiotic, such as vancomycin or the homolog of vancomycin may be administered simultaneously, e.g. the agent may be covalently attached to vancomycin or the homolog of vancomycin.

10

In yet another embodiment, this invention provides a method for determining whether a test compound selectively cleaves an ester bond present between an amino acid and an α -hydroxy carboxylic acid in a depsipeptide which
15 comprises contacting a compound of the structure X-Y, where X is an amino acid and Y is α -hydroxy carboxylic acid and - is an ester bond, with the test compound and determining whether the test compound cleaves the ester bond.

20

In yet another embodiment, the invention provides a method for determining whether a test compound selectively cleaves an ester bond present between two amino acid-like moieties in a depsipeptide which comprises contacting a
25 compound comprising the structure X-Y, wherein each of X and Y are amino acid-like moieties and - is an ester bond with the test compound and determining whether the test compound cleaves the ester bond, for example where the ester bond is present in the structure D-Ala-D-Lac.

30

In an embodiment of invention, the compound comprises the structure L-(X-Y) wherein (X-Y) is D-Ala-D-Lac, and wherein L is a detectable label, for example a dye.

- 5 In one embodiment of the invention, the test compound is bound to a solid support.

In yet another embodiment of the invention, the test compound is present in a collection of compounds
10 containing nucleophiles, for example, a combinatorial library of compounds.

As used herein "homolog of vancomycin" refers to vancomycin having at least one more CH₂ or alkene group in
15 its molecule than the vancomycin molecule. See for example U.S. Patent No. 6,037,447, the contents of which are hereby incorporated by reference into this application.

20 As used herein "glycopeptide antibiotic" refers to a class of compounds disclosed in, by way of example and not as a limitation to the present invention, U.S. Patent No. 5,977,062, the contents of which are hereby incorporated by reference into this application. For example,
25 glycopeptide antibiotics could include vancomycin as disclosed in U.S. Pat. 3,067,099; A82846A, A82846B, and A82846C as disclosed in U.S. Pat. 5,312,738; PA-42867 factors A, C, and D as disclosed in U.S. Pat. 4,946,941; A83850 as disclosed in U.S. Pat. No. 5,187,082; avoparcin
30 as disclosed in U.S. Pat. 4,322,343; actinoidin, also known as K288 (J. Antibiotics Series A 14:141 (1961)); helevecardin (Chem. Abstracts 110:17188 (1989); galacardin

(Chem. Abstracts 110:17188 (1989); and M47767 (PCT International Application No. WO 91/06566).

Organisms intrinsically resistant to vancomycin usually
5 produce D-Ala-D-Lac. Theoretically, SProC5 alone could be
bactericidal against such bacteria(e.g., Leuconostoc,
Pediococcus, Lactobacillus and Erysipelothrix sp.) (34).

The following description and examples are presented to
10 further illustrate and explain the present invention and
should not be taken as limiting in any regard. Unless
otherwise indicated in the examples and elsewhere in the
specification and claims, all parts and percentages are by
weight. Temperatures are in degrees Centigrade.

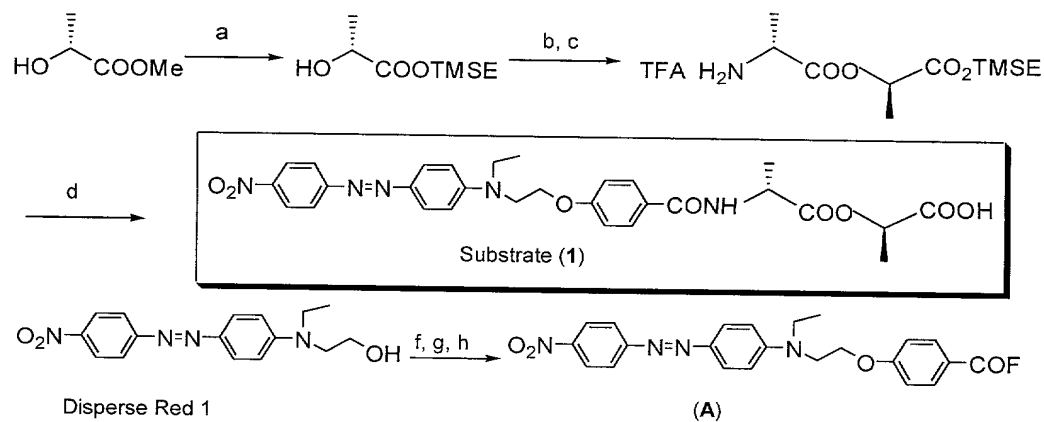
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Patented Apr 24, 1991

EXPERIMENTAL DETAILS

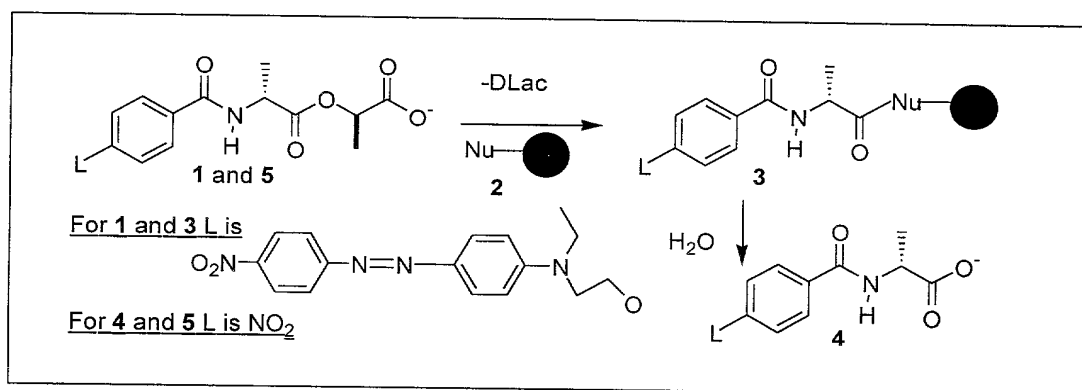
Combinatorial library screening

To find small molecules that cleave the D-Ala-D-Lac
 5 depsipeptides, the red dye-labeled analog **1** is prepared as
 a probe (Scheme 1).



Scheme 1. a. 2-(Trimethylsilyl)ethanol, $\text{Ti}(i\text{-PrO})_4$, THF; b.
 BocD-AlaCOF, DIEA, DMAP, DCM; c. TFA:DCM=4:1; d. A, DIEA,
 DMAP, DCM; e. TBAF; f. Methyl 4-hydroxybenzoate, DEAD,
 10 PPh_3 , toluene:DCM=5:1; g. LiOH; h. Cyanuric Fluoride,
 pyridine, DCM.

Substrate **1** is treated with combinatorial libraries of
 potential nucleophiles on solid phase synthesis beads (**2**),
 15 and then those library members are selected that
 covalently linked the dye to a bead (**3**). This could be
 possible assuming that noncovalently bound material would
 be washed away with a polar solvent (Scheme 2).



Scheme 2. Strategy for active sequence determination.

The libraries used for screening (i.e., the libraries used in assays were described in: (a) M. Burger, W.C. Still, *J. Org. Chem.* **60**, 7382 (1995); (b) A. Borchardt, W.C. Still, *J. Am. Chem. Soc.* **116**, 373 (1994). (c) Y. Cheng, T. Suenaga, W.C. Still, *J. Am. Chem. Soc.* **118**, 1813 (1996). (d) H. Wenemers, thesis, Columbia University (1996); (e) G. Li, thesis, Columbia University (1993); (f) E. J. Iorio, thesis, Columbia University (1999)), are presented in Figure 1 and were randomly selected from the Still group archive to achieve higher structural diversity. All libraries possess amino acid building blocks, however, the variance rises from the scaffold that allows a structurally different organization of these building blocks. All peptides were side chain deprotected and washed with DCM/TEA after TFA deprotection to ensure that all possible nucleophiles were in the unprotonated state.

The assay for screening these libraries against **1** involves shaking a desired amount of library beads with the solution of labeled substrate for a period of time. To the library beads were added 200μL solution of substrate **1** in

12DCE (or DMF), and the mixture was rotated for 3-5 days in a small glass vial. DMF was added and shaking continued for 1h. Solvent was removed and the washing was repeated several times. One wash was performed with one drop of benzylamine added to DMF to remove any remaining physically bound substrate. The strongly red beads were picked individually in 1.5 μ L DMF (in 25 μ L capillaries) and photolysed for 6h under a short wave UV lamp. Decoding was achieved by injecting the content of each capillary in EC-GC and comparing the chromatogram to a standard (20,21).

After the reaction occurred, the label-carrying beads were selected and analyzed (20,21). The initial assays were performed in 1,2-dichloroethane (12DCE) at a concentration of 2.3mM in substrate 1. Results revealed that in every library the active sequences carried serine at the amino-terminal position. This finding is remarkable considering that other nucleophiles such as Thr, Lys and terminal amino functionality, present in the screened libraries, did not appear at that position in the red beads.

Additionally:

- for library GLPro, 80% of the active beads carried the sequence D-Pro-L-Pro-L-Ser on the first arm (C3), while the other 20% Gly-L-Pro-L-Ser on the second arm (C7)
- library MB3 showed activity only after previous equilibration with $\text{Cu}(\text{OAc})_2$. Position A_2A_3 was always occupied by the sequence Pro-Ser, while A_1 was somehow variable

- library MB4 revealed only one active sequence: D-Asn-L-Lys-L-Pro-L-SerNH₂
 - library SSY carried exclusively the dimethylurea capping group from a choice of 14 others, and the most colored beads had frequently Pro and Lys in a neighboring position to the terminal Ser
 - library Yuan Cheng exhibited activity only if initially equilibrated with Cu(OAc)₂. Under those conditions, 85% of the active beads had the calibration mark: *trans* L-hydroxyPro1-A₁-A₂ *cis,trans* -L-hydroxyPro2-D-Ser-A₄
 - libraries HW and JW, acetylated tripeptide libraries, although carrying a larger selection of amino acids than SSY, showed no activity under the assay conditions
- Control assays were performed with side chain protected libraries, and additionally, with the trimethylsilyl ethyl ester of the substrate 1. Neither assay resulted in active sequences.
- To improve selectivity in the case of library SSY, assays were performed in different solvents and lower concentrations. From a choice of 12DCE, DCM, THF and DMF, best results were obtained in DMF where selectivity and intensity of the beads were enhanced. A decrease in concentration of the substrate to 0.85mM also improved selectivity. This concentration proved to be the lower threshold for eye detection of red beads.

Under these conditions, three sequences were most prominently found:

- X-L-Lys-L-Ser dimethylurea
- X-D-Lys-D-Ser dimethylurea where X was variable and
- 5 ■ L-Lys-D-Pro-L-Ser dimethylurea

After a careful analysis of the results, one can speculate that all active sequences carry a nucleophile (Ser) and an electrophile (Lys in most cases, Cu^{2+} for libraries Yuan
10 Chen and MB3, probably a backbone NH for library GLPro). These must be effectively oriented to make the nucleophilic attack of serine possible. The prevalence of Pro suggests that this amino acid may be involved in inducing conformational rigidity and therefore, pre-
15 organization of the active sites.

Computer Modeling of the Active Peptides

To gain a better understanding of why these sequences were favored and how they work, molecular modeling studies were
20 carried out on selected sequences and on the complexes of these sequences with D-Ala-D-Lac (Figure 2). Simulations were performed using the GB/SA solvation method for water and AMBER* force field, as implemented in Macromodel V6.0. We used MCMM (Monte Carlo Multiple Minimum) alternated
25 with LMCS (Low Frequency Mode Conformational Search) as conformational search methods. We found that MCMM performed better for finding minima different from the initial conformations, while LMCS for minima close to the initial conformations. Generally, a search was started
30 with MCMM, the output conformations were re-minimized and the conformations lower than 3 or 5 kcal were used as input for a new search using LMCS until convergence was

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obtained. This method was applied for the sequence BnNHL-Lys-D-Pro-L-Ser dimethylurea (**4a**), an active sequence from library SSY.

- 5 Modeling supports the observations deduced from the combinatorial assays. The structural skeleton permits Lys and Ser to be in close proximity. Lys is involved in binding the carboxylate of D-Ala-D-Lac in addition to its role as the electrophile that stabilizes the tetrahedral transition state. Additionally, the nucleophilicity of Ser is enhanced by hydrogen bonding to the urea capping group. This explains why the dimethyl urea capping group (the best hydrogen acceptor) was the only one that occurred in the active sequences resulted from the library SSY.
- 10
- 15 Furthermore, the hydroxyl is favorably positioned for attacking the ester group of the depsipeptide.

Study of the electivity and efficiency of these peptides in cleaving D-Ala-D-Lac

- 20 To test the efficiency of these simple peptides in cleaving D-Ala-D-Lac under physiologically relevant conditions, we chose studying L-Lys-D-Pro-L-Ser dimethylurea, an active sequence found more frequently in the assays.

- 25 The peptide **4a** and the depsipeptide derivative **5** were prepared in solution (Figure 3 and Scheme 2). The need to replace the substrate **1** with **5** emerged because of the poor solubility of **1** in water.

The ability of peptide **4a** in cleaving the substrate **5** was assessed in aqueous phosphate buffer pH=7 at 37°C. Stock solutions of 16mM of **5** and 130mM of peptide were made in water. Phosphate buffer solution of 25mM was made by
5 adjusting the pH of a K₂HPO₄ solution to 7.0 by addition of concentrated HCl. To a 1.5mL glass vial (Waters) were added 5μL stock solution of **5** and 15μL stock solution of peptide, followed by 135μL buffer. The volume was adjusted to 160μL by addition of water. Vials were kept in an
10 incubator at 37°C and 2μL aliquots were taken every 3h. Each aliquot was diluted with water to 5μL and 2μL were injected in HPLC. Separation of components was carried out on a reverse phase column C18 (Waters) using a gradient water / acetonitrile (0.1% TFA).

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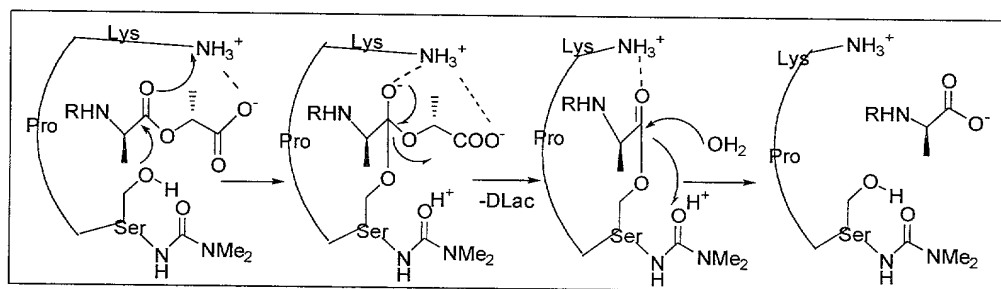
Using HPLC and monitoring the *p*-NO₂-phenyl derivative by UV at 275nm, we could easily follow the disappearance of D-Ala-D-Lac derivative **5** and the formation of the hydrolyzed product **4**. Under these conditions, we observed a 20%
20 cleavage of the depsipeptide in 24hrs. No significant effect on the rate of hydrolysis over buffer was observed using the control sequences **6**, **7**, **8** alone or **6** and **8** combined (Figure 4). This proves that the whole structural assembly is necessary for the reaction to occur and that
25 the reaction is not an artifact resulted by a change in the pH of the media due to the presence of the amine functionality.

30

The enantiomer of **4a** was synthesized through the same procedure and its ability to cleave the depsipeptide was measured. The data obtained (not shown) suggests that the enantiomer is less than half as active as **4a**. This observation explains why the enantiomeric peptide D-Lys-L-Pro-D-Ser dimethylurea was never found as an active sequence in the combinatorial library assays. The presence of a well-oriented assembly of a nucleophile and electrophile is essential but not sufficient for the reaction to occur; chiral complementary between the depsipeptide and the cleaving molecule is also required and suggests the formation of a complex between the two molecules prior to the cleavage of the ester.

15 Mechanistic studies

To prove that the reaction occurs via a nucleophilic attack by serine, the cleavage of **1** by **4a** was studied in THF-5% water, media in which the transesterification product **3** could be observed (Scheme 3).



20 **Scheme 3.** Proposed mechanism for the catalytic cleavage of D-Ala-D-Lac by **4a**.

The reaction could be monitored by HPLC at 485nm, and the separation of the three components was easily performed on an analytical reverse phase column using a gradient of

25

acetonitrile : water. Stock solutions of 2mM concentration of **1** in THF and 49mM of **4a** (lyophilized from PIPES buffer pH=7.0) in water were prepared. In three ampoules were added 40μL solution of **1**, 7μL of **4a** and the volume was
5 adjusted to 160μL with THF. For background measurements, in another three ampoules 7μL water were added instead of **4a** to the solution of **1**. All six ampoules were sealed under an argon stream and placed in an oil bath heated at 60°C. For each measurement one vial was opened and 5μL were
10 taken, diluted with 5μL THF and 2μL of this solution were injected in the HPLC. Isolation of the intermediate **3** proved to be however, more difficult. Application of the assay mixture to a size exclusion column (Sephadex LH-20 with DMF) gave a fraction enriched in **3**, and this was used
15 for a COSY-¹H NMR analysis (Figure 16). Comparison of the NMR spectra of **4a**, **3** and **9** confirmed the identity of the intermediate **3** (Figure 17). Mass spectrum analysis (MS) was used to additionally establish the identity of the three peaks seen in the HPLC chromatogram (Figure 15).

20

Small molecule development

The peptides resulted from the non-biased combinatorial libraries screenings are not useful as therapeutic agents due to their low catalytic activity. Additionally, they
25 are easily destroyed by proteases. The goal of such screens was to gain an understanding of the key elements required for selective and catalytic cleavage of the altered termini and then assemble them in a simple structure.

30

If our observations are correct, a small molecule that has a hydroxyl (serine-like functionality) of enhanced

nucleophilicity, a well-oriented electrophile and a complementary chirality to the depsipeptide should catalyze the cleavage of the D-Ala-D-Lac with an efficacy comparable to the small peptide **4a**. N-acylated prolinol derivatives (Figure 5) are the simplest structures that could fulfill such requirements. Their primary alcohol functionality forms an internal H-bond with the amide, the structures allow for the addition of the electrophile (NH₂) through various linkers and moreover, are chiral molecules. The derivatives were tested for their ability to cleave **5** in aqueous phosphate buffer pH=7.0 at 37°C (Figure 6). Addition of 12mM **SProC5** induces the cleavage of 50% of depsipeptide in 24hrs, implying that the **SProC5** derivative is twice as active as the initial peptide **4a**. One explanation for the higher activity of the very simple molecule **SProC5** compared to **4a** is the enhanced nucleophilicity of its hydroxyl (H position in H¹ NMR 7.59ppm vs 6.62ppm). Activity declines in the series with the decrease of the chain length from 5 carbons to 1 carbon. This result can be explained not only by a decrease in efficiency of the terminal amino in reaching to the carboxylate and ester of D-Ala-D-Lac with the shortening of the chain, but also by the decrease in nucleophilicity of the hydroxyl. A 6 carbon chain is also less active. The lower activity of **SProC6** is probably due to the higher flexibility of the carbon chain that does not render the amino group available for the reaction. To confirm these speculations we compared the chemical shifts of the OH and NH in the H¹ NMR spectra of the NHBoc protected SProCn series (Table 1).

Substrate **5** was used at 0.5mM, while prolinol derivatives were run at 12mM. The graph is the average of three separate assays (Figure 6).

- 5 The study shows that there is a competition between the amino group and the hydroxyl for hydrogen bonding to the amide. With the decrease of the chain length, the probability of the OH being hydrogen bonded decreases substantially, fact reflected in the chemical shifts of
10 the OH and NH with the change in the length of the carbon chain.

Boc- derivative	OH position (ppm)	NH position (ppm)
SProC1	4.57	5.48
SProC2	4.98	5.28
SProC3	4.96	4.75
SProC4	5.11	4.65
SProC5	5.14	4.54
SProC6	5.28	4.52
SProUC4	4.86	4.65

15

Table 1. Shifts in the position of OH and NHBoc with the modification of the carbon chain length.

The table also explains the low activity of **SProUC4**,
20 derivative in which the amide is replaced by urea. The urea is a better acceptor than the amide and should increase the reactivity of the hydroxyl. However, it is

possible that structural constraints imposed by the urea play an important role and do not allow for proper orientation for H-bonding.

5 Molecular modeling performed on the complex of **SProC5** and PhD-Ala-D-Lac additionally confirms the structural fit of this small molecule for the cleavage of the depsipeptide (Figure 7).

10 **In vivo testing of the small molecules against VRE**

The designed small molecules should theoretically enhance the biological activity of vancomycin by reducing the pool of C-terminal altered cell wall precursors in bacteria that have intrinsic or acquired resistance to vancomycin.

15 Therefore, to test the activity of the synthetic molecules, we used as reference organism a *vanA* enterococci, strain EF228 (22), and for comparison, the susceptible reference strain enterococci JH2-2 (23). **SProC5**, the best candidate molecule was studied in

20 combination with vancomycin. *Enterococcus faecium* EF228 (22), a *vanA* strain and *Enterococcus faecalis* strains JH2-2 (23) were grown on BHI (Difco or Oxoid) agar or broth at 37°C. Biological assays were performed in 96-well tissue culture plates (MICROTEST U-bottom, Falcon, Becton

25 Dickinson). A range of vancomycin (Sigma) concentrations (100 µl per well) were used based on sequential two-fold dilution starting from 100 and 2000 µg/ml to 0.1 and 1.96 µg/ml for strains JH2-2 and EF228, respectively. The different molecules were added (100 µl per well) at fixed

30 concentrations (0, 5, 10, 50 and 100 mM) containing an inoculum of either strain at a final dilution of 10⁻² obtained from an overnight culture. The range of effective

vancomycin concentrations started at 50 and 1000 µg/ml for JH2-2 and EF228, respectively. Micro-titer plates were incubated at 37°C without agitation for 18 hours. Cell sediments were resuspended by shaking and optical density at 600nm was measured with an ELISA Multiskan RC plate reader (Labsystems, Helsinki, Finland). Bactericidal activity was determined by serially diluting (10^{-2} , 10^{-4} and 10^{-5}) each well in BHI broth and plating 10 µl of each dilution on BHI agar plates. Plates were incubated 24 hours and the number of colony forming units per ml was determined.

A reduction of 10% in the load of altered termini should theoretically decrease the MIC of vancomycin by 10-fold.

Figure 8 illustrates the specific synergistic effect of **SProC5** against the *vanA* strain EF228. Panel A shows that vancomycin alone inhibited growth at 500 µg/ml while **SProC5** (50 mM) combined with vancomycin reduced the minimum inhibitory concentration to 62.5 µg/ml, i.e. a 8-16 folds decrease in the MIC. Bactericidal activity was confirmed by determining the number of cells that survived the combined treatment of vancomycin and **SProC5** (Figure 8A, black bars). Indeed, the combination of 62.5 µg/ml of vancomycin with 50 mM of **SProC5** resulted in a three log decrease in bacterial load compared to vancomycin or **SProC5** alone. This value was increase to four log when 250 µg/ml of vancomycin was used with 50 mM **SProC5**. The synergistic effect of **SProC5** was dose dependent as shown in Figure 8B (5 mM of **SProC5** was ineffective while 10 mM had an intermediate activity).

Specificity of **SProC5** mode of action against D-Ala-D-Lac
termini is reinforced by the complete absence of increased
sensibility of JH2-2 to vancomycin by **SProC5**, **SProC2** or
any of the control molecules, even at 100 mM. Results
5 represent the average of two independent experiments
(Figure 8)

SProC5 alone had no inhibitory or bactericidal activity
10 against enterococci (see Figure 8). Its synergistic effect
with vancomycin could be related to a distinct mechanism
than the one predicted based on the specific hydrolytic
activity of **SProC5**. To validate the mechanism, we compared
SProC5 activity to a related molecule **SProC2** characterized
15 by a lower D-Ala-D-Lac hydrolytic activity in our kinetic
assays (see Figure 6). Indeed, as predicted from the
hydrolytic activity, **SProC2** had a much lower synergistic
effect with vancomycin although not negligible (at 50 mM,
MIC to vancomycin decreased to 250 µg/ml). **SProC2** was able
20 to decrease the MIC to vancomycin only by 2 to 4 folds in
the conditions tested (see Figure 8B).

Further evidence correlates the hydrolytic activity of
SProC5 with its synergy with vancomycin. **SProC5** activity
25 was compared to that of its enantiomer molecule **RProC5** and
to its corresponding 5 carbon unit (**C5**). None of the
control molecules had a synergistic effect with vancomycin
even at 100 mM (see Figure 8C) strongly suggesting that
the basis for the biological activity of **SProC5** was
30 derived from its enhanced and specific D-Ala-D-Lac
hydrolytic activity.

Finally, strain JH2-2 which is susceptible to vancomycin and does not synthesize altered cell wall precursors, was used as a control. The sensitivity of JH2-2 to vancomycin was unaffected by the presence of any of the synthetic molecules (**SProC2**, **C5**, **RProC5** and **SProC5**) even at 100 mM (Figure 8D). MIC of vancomycin was unchanged in any of the tested conditions (MIC between 1.58 and 3.13 µg/ml).

Through screening of combinatorial libraries and the study of the active sequences and patterns, an understanding of the essential key elements for catalytic and selective cleavage of D-Ala-D-Lac is achieved. Assimilation of these features resulted into the design of a simple small molecule that is more effective in cleaving the depsipeptide than its paternal small peptide **4a**. This molecule, **SProC5**, increases the sensitivity of vanA resistant bacterial strain to vancomycin. Our in vivo results suggest that **SProC5** enhances vancomycin's activity because of its D-Ala-D-Lac's hydrolytic activity. The synergistic effect of **SProC5** mirrors its ability to hydrolyze the depsipeptide bond. Several observations support this hypothesis. First, a related compound **SProC2**, which has a lower hydrolytic activity, is less efficient in enhancing vancomycin's inhibitory activity as predicted from the kinetic studies. Furthermore, the enantiomer of **SProC5** - **RProC5** - lacks any synergy with vancomycin. Finally, the enterococcal strain JH2-2 which is unable to synthesize altered cell wall precursors was completely insensitive to the activity of the synthetic molecules. Taken together, these data suggest that the mechanism of synergy in vivo is based on the hydrolytic capability of the synthetic compound **SProC5**.

SProC5 can reverse resistance in several ways. It could similarly as VanX to reduce the cytoplasmic pool of D-Ala-D-Lac at every step of the biosynthetic pathway, therefore
5 favoring the synthesis of normal cell wall precursors terminating in D-Ala-D-Ala.

Alternatively, **SProC5** could remain extracellular and actively hydrolyze cell wall lipid-intermediates. Lipid
10 intermediates would be truncated as disaccharide-tetrapeptides, an accumulation which would result progressively in a hypocrosslinked peptidoglycan. *E.coli* mutants that are unable to properly recycle tetrapeptide turnover products, accumulate tetrapeptide derivatives
15 resulting in lysis and death in stationary phase (24).

A third mechanism can be envisioned. The resistance phenotype in *vanA* strains is inducible and dependent on a two-component regulatory system. VanS and VanR function as
20 a sensor and a response regulator, respectively. The suggested signal sensed by VanS appears be the accumulation of lipid II (undecaprenyl-disaccharide-pentatpeptide) (25-27). The accumulation of tetrapeptide derivatives of lipid II due to **SProC5** hydrolytic activity
25 might compete for the binding site of VanS interfering with the signaling cascade and the induction of *vanHAXYZ* transcription.

The three hypotheses for the mode of action of **SProC5** are
30 not exclusive and could occur at the same time. A combination of muropeptide, cell wall precursor composition analysis and transcription analysis of the

vanA cluster would be needed to distinguish between the different mechanisms.

Molecules that catalytically and selectively cleave the altered termini of the bacteria cell wall can disable the antibiotic-resistance mechanism in these pathogens. The molecules act by re-sensitizing bacteria to the drug and could be used in concert with vancomycin in the treatment of VRE. Additionally, this work shows that bits of information obtained from the screening of non-biased random libraries and from molecular modeling can be assimilated in the design of structurally different molecules that act by the same mechanism. One can envision that a more potent candidate for the cleavage of the D-Ala-D-Lac could result from screening biased libraries that assemble the structural characteristics described in this work. We believe the approach for identifying novel molecules able to enhance the activity of vancomycin has long term potential for the management of infectious diseases.

Combinatorial library assay development

The libraries used in screening for D-Ala-D-Lac cleavers are presented in Figure 1 and were randomly selected from the Still group archive to achieve higher structural diversity. All libraries possess amino acid building blocks, however, the variance rises from the scaffold that undoubtedly allows a structurally different organization of these building blocks. Library GLPro (28) uses 12-deoxycholic acid scaffold that allows differential derivatization of the two arms due to the distinct reactivity of the C3 and C7 hydroxyls, while library Yuan

Chen (29) employs, in addition to the cholic acid core, derivatized hydroxyprolines to which the amino acids are linked. The cyclen core scaffold used for libraries MB3 and MB4 (30) contains three NH's as starting points for
5 growing the identical three or four amino acid peptide chains, respectively. Libraries SSY (31), HW (32) and JW (33) are acylated tripeptide libraries linked to the solid support through a caproic acid unit. However, library SSY contains 15 different acylating groups, while all the
10 members of libraries HW and JW are acetylated. All peptides were side chain deprotected and washed with dichloromethane (DCM)/ triethylamine (TEA) after trifluoroacetic acid (TFA) deprotection to ensure that all possible nucleophiles were in the unprotonated state.

15 The assay for screening these libraries against **1** involved shaking a desired amount of library beads with the solution of labeled substrate for a period of time. After the reaction occurred, the label-carrying beads were
20 selected and analyzed. The initial assays were performed in 1,2-dichloroethane (12DCE) at a concentration of 2.3 mM in substrate **1**. After 3 days of shaking, the beads were extensively washed with dimethylformamide (DMF) and once with a diluted solution of benzylamine in DMF to remove
25 any physically bound substrate (Figures 9A and 9B).

To improve selectivity in the case of library SSY, assays were performed in different solvents and lower concentrations. From a choice of DCE, DCM, tetrahydrofuran
30 (THF) and DMF, best results were obtained in DMF where selectivity and intensity of the beads were enhanced (Figure 11). A decrease in concentration of the substrate

to 0.85 mM also improved selectivity. This concentration proved to be the lower threshold for eye detection of red beads.

5 Results revealed that in every library the active sequences carried serine at the amino-terminal position. This finding is remarkable considering that other nucleophiles such as Thr, Lys and terminal amino functionality, present in the screened libraries, did not
10 appear at that position in the red beads.

Additionally:

- for library GLPro, 80% of the active beads carried the sequence D-Pro-L-Pro-L-Ser on the first arm (C3), while
15 the other 20% Gly-L-Pro-L-Ser on the second arm (C7)
- library MB3 showed activity only after previous equilibration with $\text{Cu}(\text{OAc})_2$. Position A_2A_3 was always occupied by the sequence Pro-Ser, while A_1 was somehow variable
- 20 - library MB4 revealed only one active sequence: D-Asn-L-Lys-L-Pro-L-SerNH₂
- library SSY carried exclusively the dimethylurea capping group from a choice of 14 others, and the most colored beads had frequently Pro and Lys in a neighboring position
25 to the terminal Ser
- library Yuan Cheng exhibited activity only if initially equilibrated with $\text{Cu}(\text{OAc})_2$. Under those conditions, 85% of the active beads had the calibration mark: trans L-hydroxyPro1- $\text{A}_1\text{-A}_2$ cis,trans-L-hydroxyPro2-D-Ser- A_4
- 30 - libraries HW and JW, acetylated tripeptide libraries, although carrying a larger selection of amino acids than SSY, showed no activity under the assay conditions

Control assays were performed with side chain protected libraries, and additionally, with the trimethylsilyl ethyl ester (TMSE) of the substrate **1**. Neither assay resulted in active sequences.

5

Combinatorial assay sequence data

Table 2. Library GLPro. 0.5 copies were used (about 10,000 beads) for a 3 days assay in 12DCE with substrate **1**. 15 red beads found.

L, DPro	A ₁	A ₂	A ₃	A ₄
DPro	Pro	Ser	Thr	Lys
DPro	Pro	Ser	Lys	Ala
DPro	Pro	Ser	Thr	Pro
DPro	Pro	Ser	Thr	Ser
DPro	Pro	Ser	Lys	Val
DPro	Pro	Ser	Pro	Val
DPro	Pro	Ser	Val	Ser
DPro	Pro	Ser	Thr	Pro
DPro	Pro	Ser	Phe	Ser
DPro	Pro	Ser	Lys	Ala
DPro	Pro	Ser	Pro	Ser
DPro	Pro	Ser	Pro	Ser
DPro	Thr	Pro	Pro	Ser
DPro	Phe	Ser	Pro	Ser
LPro	Leu	Ala	Pro	Ser

10

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Table 3. Library MB3. 0.1 copies were used, about 900 beads pre-equilibrated with $\text{Cu}(\text{OAc})_2$, in an assay performed in 12DCE with substrate 1. 7 red beads found.

5

A_1	A_2	A_3
LAla	DPro	DSer
LVal	DPro	DSer
LPro	LPro	LSer
DAsn	DPro	LSer
LVal	DPro	LSer
LAla	LPro	LSer
LGln	DPro	DSer

Table 4. Library MB4. 0.07 copies (about 9,000 beads) used for an assay performed in DMF with substrate 1. 1 red bead found.

10

A_1	A_2	A_3	A_4
DAsn	LLys	LPro	LSer

15 **Tables 5A-5C.** Library SSY. (A) 0.2 copies used, about 10,000 beads, for an assay in 12DCE with substrate 1. (B) 0.2 copies used, about 10,000 beads, for an assay in DMF with substrate 1. (C) 1.5 copies used, about 80,000 beads,

for an assay in DMF with 0.85mM substrate **1**. Only the very strongly red beads picked.

5 **Table 5A**

A ₁	A ₂	A ₃	CAP
LLys	DPro	LSer	NMe₂
LSer	LLys	LSer	NMe₂
DPro	LLys	LSer	NMe₂
DSer	LLys	LSer	NMe₂
LPro	LLys	LSer	NMe₂
LAla	DLys	DSer	NMe₂
DPro	LPro	DSer	NMe₂
LLys	LAla	LSer	NMe₂
DLys	LVal	DSer	NMe₂
LLys	DAla	LSer	NMe₂
DLys	LAla	LSer	NMe₂

10

15

20

Table 5B

A ₁	A ₂	A ₃	CAP
LLys	DPro	LSer	NMe₂
LAsn	DPro	LSer	NMe₂
LLys	LPro	DSer	NMe₂
LPro	LLys	LSer	NMe₂
DGln	LLys	LSer	NMe₂
LSer	LLys	LSer	NMe₂
LLys	DLys	DSer	NMe₂
LAla	DLys	Dser	NMe₂

TABLE 5B

Table 5C

A ₁	A ₂	A ₃	CAP	Nr. of times found
LLys	DPro	LSer	NMe₂	2
DAsn	LLys	LSer	NMe₂	3
DPro	LLys	LSer	NMe₂	1
LAsn	LLys	LSer	NMe₂	1
DSer	LLys	LSer	NMe₂	2
LPro	LLys	LSer	NMe₂	1
DAla	LLys	LSer	NMe₂	1
LVal	DLys	DSer	NMe₂	1
LAsn	DLys	DSer	NMe₂	1
DPro	DLys	DSer	NMe₂	1
DPro	DAsn	DSer	NMe₂	1
LPro	DGln	DSer	NMe₂	1

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Table 6. Library Yuan Chen. 0.2 copies used, about 8,000 beads pre-equilibrated with $\text{Cu}(\text{OAc})_2$, for an assay done in 12DCE with 1.17 red beads found.

Hp1	A ₁	A ₂	Hp2	A ₃	A ₄
trans L	DPhe	LPro	cis L	DSer	DAla
trans L	DPro	LPhe	cis L	DSer	LPro
trans L	DPro	DSer	trans L	DSer	LPhe
trans L	DPhe	DAsn	trans L	DSer	LPro
trans L	DAla	LPro	cis L	DSer	DPro
trans L	LAsn	DPro	cis L	DSer	LAla
trans L	LPro	LPhe	cis L	DSer	LAla
trans L	DPro	LPhe	cis L	DSer	LPro
trans L	DSer	LAla	trans L	DSer	LPhe
trans L	DSer	LAsn	trans L	DSer	DPro
trans L	DAla	LAla	trans L	DSer	DSer
trans L	DAla	DPro	trans L	DSer	DSer
trans L	LAla	DPhe	trans L	DSer	LAla
cis L	DAla	DSer	trans L	DSer	DSer
cis L	LSer	DSer	trans D	DAla	DSer
trans D	LPro	DPro	cis L	DSer	DSer

General synthetic procedures

All compounds were synthesized using standard laboratory techniques. Commercially available reagents and solvents were used without further purification. Reactions were
5 monitored using thin-layer liquid chromatography (TLC) and visualization was done using UV light, cerium ammonium molybdate (CAM) and permanganate.

Nuclear magnetic resonance (NMR) spectra were recorded on
10 a Varian VXR-400, Bruker 400 MHz or Bruker 300 MHz. Mass spectra were obtained with Jeol JMS-HX110A Mass Spectrometer and RIBERMAG R10-10 C. Gas chromatography was conducted with a HP 5890 GC equipped with an electron capture detector and HP ULTRA I fused silica capillary
15 column. HPLC was directed with a Waters Millennium system, using a Nova Pak C18 column.

General procedure for the synthesis of acid fluorides:

To a 1 mmol solution of acid in 15 mL DCM at r.t. were
20 added 1 eq. pyridine and 1.5 eq. cyanuric fluoride. The resulting solution was stirred under argon for 1.5 h. After dilution with 150 mL DCM, the organic layer was washed with 2 mL water. The solvent was removed to give the acid fluorides, which were used without further
25 purification.

Figure 11. Step (a) Synthesis of R(+)-2-(Trimethylsilyl)ethyl lactate. A solution of 460 μ L (4.8 mmols) of R-methyl lactate, 1.6 mL (5.38 mmols) $\text{Ti}(\text{i-PrO})_4$ and 820 μ L (5.7 mmols) 2-(trimethylsilyl)ethanol in 30 mL
30 dry THF was refluxed under argon overnight. Solvent was

removed. Purification on column chromatography (DCM:acetone at 8:1) gave 700 mg of product in 85% yield.

¹H NMR (400 MHz, CDCl₃): δ4.28 (m, 2H), 4.22 (m, 1H), 2.82 (d, *J* = 3.0 Hz, 1H), 1.41 (d, *J* = 7.3 Hz, 3H), 1.02 (m, 2H), 0.05 (s, 9H). Chemical Impact Mass Spectrum (CIMS) (NH₃): *M* = 190 calculated for C₈H₁₈O₃. Found *m/z* = 191 (*M*+1).

Figure 11. Steps (b, c). Synthesis of D-Ala-R(+)-2-(Trimethylsilyl)ethyl lactate. To a solution of 700 mg (3.68 mmols) D-LacTMSE in 15 mL DCM, were added 800 mg (4.2 mmols) of the acid fluoride of D-AlaBoc, 1.2 mL (7.4 mmols) *N,N*-diisopropylethylamine (DIEA) and a catalytic amount of dimethylaminopyridine (DMAP). The mixture was stirred at r.t. under argon for 3 h. Solvent was removed, and purification on column chromatography (DCM:Petroleum ether:EtOAc at 10:4:1) gave 900 mg of BocD-Ala-R(+)-2-(Trimethylsilyl)ethyl lactate (90% yield).

¹H NMR (400 MHz, CDCl₃): δ5.15-5.08 (m, 1H), 5.05-4.97 (bd, 1H), 4.42-4.37 (m, 1H), 4.27-4.19 (m, 2H), 1.51 (d, *J* = 7.1 Hz, 3H) 1.47 (d, *J* = 7.3 Hz, 3H), 1.45 (s, 9H) 1.05-0.96 (m, 2H), 0.05 (s, 9H). ¹³C NMR (300 MHz, CDCl₃): δ173.3, 170.9, 155.5, 80.3, 69.6, 64.3, 49.4, 28.7, 18.9, 17.7, 17.2, -1.1. CIMS (NH₃): *M* = 361 calculated for C₁₆H₃₁O₆NSi. Found *m/z* = 362 (*M*+1).

To this material were added 15 mL mixture DCM:TFA at 3:1, and the solution was stirred at r.t. for 15 min. After solvent removal, the TFA salt was used in the next step

without further purification.

Figure 11. Steps (f, g). A solution of 400 mg (1.26 mmols) Disperse Red 1, 232 mg (1.52 mmols) Methyl-(4-hydroxy) benzoate, 434 mg (1.76 mmols) PPh₃ and 400 µL (2.52 mmols) diethyl azodicarboxylate (DEAD) in 25 mL toluene/5 mL DCM, was stirred overnight under argon, at r.t.. Solvent was removed. 200 mL DCM were added and the solution was washed with 3x15 mL 10% NaOH. Purification on column chromatography (DCM) gave the methyl ester as a red solid.

¹H NMR (400 MHz, CDCl₃): δ 8.34 (d, *J* = 9.1 Hz, 2H), 7.99 (d, *J* = 8.8 Hz, 2H), 7.94 (d, *J* = 9.0 Hz, 2H), 7.93 (d, *J* = 9.1 Hz, 2H), 6.92 (d, *J* = 8.8 Hz, 2H), 6.83 (d, *J* = 9.0 Hz, 2H), 4.26 (t, *J* = 5.9 Hz, 2H), 3.90 (t, *J* = 5.9 Hz, 2H), 3.89 (s, 2H), 3.63 (q, *J* = 7.1 Hz, 2H), 1.31 (t, *J* = 7.1 Hz, 3H). CIMS (NH₃): *M* = 448 calculated for C₂₄H₂₄O₅N₄. Found *m/z* = 449 (*M*+1).

To this material were added 20 mL MeOH/40 mL THF/10 mL H₂O followed by 5 equivalents of LiOH, and the solution was refluxed for 3 h. Solvent removed. After addition of 200 mL DCM, HCl concentrated solution was slowly added until the solid has dissolved. The organic layer was washed with 2x 10 mL water. Solvent was removed to give 475 mg product in 83% overall yield. This was used without further purification.

¹H NMR (400 MHz, dimethylsulfoxide-d₆ (DMSO)): δ 8.37 (d, *J* = 9.1 Hz, 2H), 7.94 (d, *J* = 9.1 Hz, 2H), 7.87 (d, *J* = 8.8 Hz, 2H), 7.85 (d, *J* = 9.0 Hz, 2H), 7.05 (d, *J* = 8.8 Hz,

2H), 6.97 (d, $J = 9.0$ Hz, 2H), 4.28 (t, $J = 5.9$ Hz, 2H),
3.92 (t, $J = 5.9$ Hz, 2H), 3.63 (q, $J = 7.1$ Hz, 2H), 1.20
(t, $J = 7.1$ Hz, 3H). ^{13}C NMR (300 MHz, DMSO): δ 168.1,
162.4, 157.1, 152.5, 147.7, 143.7, 132.2, 126.9, 125.1,
5 123.4, 118.9, 114.9, 112.6, 66.6, 49.9, 46.2, 12.9. CIMS
(NH_3): $M = 434$ calculated for $\text{C}_{23}\text{H}_{22}\text{O}_5\text{N}_4$. Found $m/z = 435$
($M+1$).

Figure 11. Step (h). To a solution of 265 mg (0.61 mmols)
10 carboxylic acid in 10 mL DCM were added 53 μL (0.61 mmols)
pyridine and 59 μL (0.8 mmols) cyanuric fluoride, and the
mixture was stirred under argon at r.t. for 1.5 h. The
solution was diluted with 150 mL DCM and washed 1x 2 mL
water. Solvent was removed to give the crude product.

15 Figure 11. Step (d) Synthesis of DR1-PhNH-D-Ala-D-Lactate-
2-(ethyl)trimethylsilyl. The acid fluoride obtained as
above, was taken in 20 mL DCM and added to the TFA salt of
the amine, followed by 190 μL (1.1 mmols) DIEA and a
20 catalytic amount of DMAP, and the solution was stirred at
r.t. under argon for 3 h. Solvent was removed and the
product was purified by column chromatography (DCM:acetone
at 15:1) to give 300 mg of red solid, in 80% yield.

25 ^1H NMR (400 MHz, CDCl_3): δ 8.33 (dd, $J = 8.0$ Hz, $J = 1.9$ Hz,
2H), 7.93 (dd, $J = 8.0$ Hz, $J = 1.9$ Hz, 2H), 7.92 (dd, $J =$
8.0 Hz, $J = 2.1$ Hz, 2H), 7.76 (dd, $J = 9.3$ Hz, $J = 2.0$ Hz,
2H), 6.92 (dd, $J = 8.0$ Hz, $J = 2.1$ Hz, 2H), 6.82 (dd, $J =$
9.3 Hz, $J = 2.0$ Hz, 2H), 6.59 (d, $J = 6.8$ Hz, 1H), 5.20-
30 5.11 (m, 1H), 4.90-4.82 (m, 1H), 4.28 (m, 4H), 3.90 (t, J
 $= 5.9$ Hz, 2H), 3.62 (q, $J = 6.9$ Hz, 2H), 1.61 (d, $J = 7.2$

Hz, 3H), 1.54 (d, $J = 7.0$ Hz, 3H), 1.29 (t, $J = 6.9$ Hz, 3H), 1.05-0.99 (m, 2H), 0.05 (s, 9H). Low resolution mass spectrum (LRMS) fast atom bombardment (FAB): $M = 677$ calculated for $C_{34}H_{43}O_8N_5Si$. Found $m/z = 678$ ($M+1$).

5

Figure 11. Step (e). Synthesis of depsipeptide analog DR1-PhNH-D-Ala-D-Lactatic acid (**1**). To a solution of 300 mg (0.44 mmols) of trimethylsilylether (TMSE) protected **1** in 15 mL DMF, were added 440 μ L (0.44 mmols) solution 1 M tetrabutylammonium fluoride (TBAF) in THF. Solution was stirred for 1 h at r.t.. After solvent removal the solid was taken in 150 mL of EtOAc, acidified with acetic acid and washed with 3x 10 mL water. The solvent was removed, and purification by gel chromatography (Sephadex LH-20) with MeOH (1% AcOH) gave 254 mg **1** as a red solid in 80% yield over the last two steps.

1H NMR (400 MHz, $CDCl_3$): δ 8.33 (dd, $J = 8.0$ Hz, $J = 1.9$ Hz, 2H), 7.93 (dd, $J = 8.0$ Hz, $J = 1.9$ Hz, 2H), 7.92 (dd, $J = 8.0$ Hz, $J = 2.1$ Hz, 2H), 7.76 (dd, $J = 9.3$ Hz, $J = 2.0$ Hz, 2H), 6.92 (dd, $J = 8.0$ Hz, $J = 2.1$ Hz, 2H), 6.82 (dd, $J = 9.3$ Hz, $J = 2.0$ Hz, 2H), 6.62 (d, $J = 6.8$ Hz, 1H), 5.24-5.15 (m, 1H), 4.86-4.78 (m, 1H), 4.24 (t, $J = 5.9$ Hz, 2H), 3.90 (t, $J = 5.9$ Hz, 2H), 3.62 (q, $J = 6.9$ Hz, 2H), 1.60 (d, $J = 7.2$ Hz, 3H), 1.59 (d, $J = 7.0$ Hz, 3H), 1.29 (t, $J = 6.9$ Hz, 3H). ^{13}C NMR (300 MHz, DMSO): 172.4, 171.6, 165.6, 160.7, 156.1, 151.5, 146.7, 142.7, 129.3, 125.9, 124.8, 122.4, 113.8, 111.6, 68.5, 65.5, 48.9, 47.9, 45.2, 16.6, 16.5, 11.9. High resolution mass spectrum (HRMS) (FAB): calculated for $C_{29}H_{31}O_8N_5$ ($M+1$) 578.2251, found 578.2255. Infrared analysis (IR) (polyethylene card):

3300, 3010, 2917, 2950, 1746, 1735, 1710, 1604, 1513, 1339, 1253.

Synthesis of 4-Nitro-PhNHD-Ala-D-Lac (5). To the TFA salt solution of D-Ala-D-LacTMSE in 10 mL DCM were added 2 equivalents of DIEA, a catalytic amount of DMAP and 1.3 equivalents of acid fluoride of the 4-nitrobenzoic acid dissolved in 10 mL DCM. The solution was stirred for 3 h at r.t. under argon. Solvent was removed and the product separated on silica gel column with DCM:hexanes:acetone at 7:2:1. Deprotection of the TMSE was performed as previously described, and after purification on Sephadex LH-20 (MeOH -1% AcOH), **5** was obtained as a white powder in 80% overall yield.

¹H NMR (400 MHz, DMSO): δ 9.17 (d, *J* = 6.8 Hz, 1H), 8.38 (d, *J* = 7.9 Hz, 2H), 8.11 (d, *J* = 7.9 Hz, 2H), 5.02-4.96 (m, 1H), 4.61-4.51 (m, 1H), 1.49 (d, *J* = 7.3 Hz, 3H), 1.43 (d, *J* = 7.0 Hz, 3H). ¹³C NMR (300 MHz, DMSO): δ 172.8, 172.2, 165.7, 150.0, 140.1, 129.8, 124.4, 69.7, 49.1, 17.6, 17.3. HRMS (FAB): Calculated for C₁₃H₁₄O₇N₂ (M+1) 311.2713, found *m/z* = 311.0887.

Synthesis of D-Ala derivative (4). The product was obtained as a white solid in the matter described above.

¹H NMR (400 MHz, DMSO): δ 12.68 (bs, 1H), 9.02 (d, *J* = 7.1 Hz, 1H), 8.31 (d, *J* = 8.7 Hz, 2H), 8.11 (d, *J* = 8.7 Hz, 2H), 4.51-4.41 (m, 1H), 1.35 (d, *J* = 7.3 Hz, 3H). ¹³C NMR (300 MHz, DMSO): δ 174.7, 165.4, 149.4, 140.4, 129.8, 124.4, 49.3, 17.6. CIMS (CH₄): *M* = 238 calculated for

$C_{10}H_{10}O_5N_2$. Found $m/z = 239$ ($M+1$).

Synthesis of peptide 4a.

BnNHL-Lys (Boc)NH₂: A solution of 470 mg (1 mmols) FmocL-Lys(Boc)OH, 230 mg (1.2 mmols) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 165 μ L (1.5 mmols) benzylamine and a catalytic amount of DMAP in 25 mL DCM, was stirred at r.t. under argon for 2 h. After dilution with 25 mL DCM, the solution was washed with 5 mL solution HCl 1 M. Solvent was removed to give the benzylamide as a white solid. This was deprotected by stirring with 50 mL solution DCM:piperidine at 4:1, at r.t. for 30 min. Separation on silica gel column with DCM:MeOH at 15:1 to 7:1, gave the amine in 90% yield (300 mg).

¹H NMR (400 MHz, CDCl₃): δ 7.71-7.59 (bt, 1H), 7.38-7.27 (m, 5H), 4.63-4.53 (bt, 1H), 4.48 (d, $J = 5.9$ Hz, 2H), 3.45-3.37 (m, 1H), 3.18-3.04 (m, 2H), 1.94-1.82 (m, 1H), 1.68-1.41 (m, 5H), 1.42 (s, 9H). CIMS (NH₃): $M = 335$ calculated for $C_{18}H_{29}O_3N_3$. Found $m/z = 336$ ($M+1$).

BnNHL-Lys (Boc) -D-ProNH₂: To the solution of amine were added 340 mg (1 mmols) acid fluoride of FmocD-Pro and 350 μ L (2 mmols) DIEA in 50 mL DCM, and stirred at r.t. under argon for 1 h. After solvent removal, purification was completed on silica gel column with DCM:acetone at 5:2. Fmoc deprotection was carried out as above. After separation on silica gel column with DCM:MeOH at 10:1 then 7:1 (1% TEA), the amine was obtained in 85% yield over the two steps.

¹H NMR (300 MHz, CDCl₃): δ8.03 (d, *J* = 8.3 Hz, 1H), 7.39-7.23 (m, 5H), 6.91-6.82 (m, 1H), 4.67-4.59 (bt, 1H), 4.57-4.31 (m, 3H), 3.81-3.72 (m, 1H), 3.17-3.04 (m, 2H), 3.02-2.74 (m, 2H), 2.18-1.41 (m, 10H), 1.42 (s, 9H). CIMS (NH₃):
5 M = 432 calculated for C₂₃H₃₆O₄N₄. Found m/z = 433 (M+1).

BnNHL-Lys (Boc) -D-Pro-L-Ser (tBut)NH₂: To the above amine in 50 mL DCM were added 380 mg (1 mmols) acid fluoride of
10 FmocL-Ser(tBut) and 350 μL (2 mmols) DIEA, and the resulting solution was stirred under argon for 1 h. After solvent removal, the purification was achieved by flash chromatography with DCM:acetone at 2:1. After Fmoc deprotection and separation on silica gel column with
15 DCM:MeOH at 14:1, the amine was obtained as 420 mg of white solid in 75% yield.

¹H NMR (300 MHz, CDCl₃): δ7.66-7.58 (bt, 1H), 7.39-7.23 (m, 5H), 6.82 (d, *J* = 8.3 Hz, 1H), 4.79-4.71 (bt, 1H), 4.71-4.27 (m, 4H), 3.88-3.71 (m, 1H), 3.65-3.13 (m, 6H), 2.31-1.46 (m, 10H), 1.46 (s, 9H), 1.15 (s, 9H). CIMS (NH₃): M =
20 575 calculated for C₃₀H₄₉O₆N₅. Found m/z = 576 (M+1).

BnNHL-Lys (Boc) -D-Pro-L-Ser (tBut) CONMe₂: Capping of the
25 serine was accomplished with excess dimethylcarbonyl chloride and DIEA, by stirring the solution with a catalytic amount of DMAP under argon for 3 h at r.t.. Purification on a silica gel column with DCM:acetone at 1:1, gave the peptide in 85% yield.

¹H NMR (300 MHz, CDCl₃): δ 7.46 (d, *J* = 8.3 Hz, 1H), 7.34-7.24 (m, 5H), 5.43-5.32 (bt, 1H), 5.12-5.02 (bt, 1H), 3.74-3.56 (m, 4H), 3.18-3.06 (m, 2H), 2.84 (s, 6H), 2.38-1.45 (m, 10H), 1.46 (s, 9H), 1.17 (s, 9H). ¹³C NMR (300
5 MHz, CDCl₃): δ 172.3, 139.1, 128.9, 127.9, 127.6, 79.5, 74.2, 62.2, 61.1, 54.1, 47.8, 43.6, 40.4, 36.5, 30.9, 29.5, 29.3, 28.9, 27.8, 24.6, 22.9. CIMS (NH₃): *M* = 646 calculated for C₃₃H₅₄O₆N₇. Found *m/z* = 647 (*M*+1).

10 **BnNHL-Lys-D-Pro-L-Ser(tBut)CONMe₂ (7)**: The Boc was selectively removed by stirring the peptide in DCM:TFA at 3:1 for 30 min at r.t.. After the excess TFA was eliminated, the peptide was purified by gel filtration on Sephadex LH-20 with DMF.

15 ¹H NMR (400 MHz, DMSO): δ 8.28 (t, *J* = 5.8 Hz, 1H), 7.82 (d, *J* = 8.2 Hz, 1H), 7.65 (bs, 3H), 7.32-7.18 (m, 5H), 6.22 (d, *J* = 6.3 Hz, 1H), 4.42-4.12 (m, 5H), 3.74-3.36 (m, 4H), 2.72 (s, 6H), 2.08-1.16 (m, 10H), 1.12 (s, 9H). LRMS
20 (FAB): *M* = 546 calculated for C₃₃H₄₆O₅N₆. Found *m/z* = 547 (*M*+1).

BnNHL-Lys-D-Pro-L-SerCONMe₂ (4a): To the Boc protected peptide in 10 mL DCM stirred under argon at 0°C, were added
25 1.3 equivalents of TiCl₄ solution 1 M in DCM. The solution was stirred for 15 min, after which 5 mL saturated aqueous NaHCO₃ solution were added. The organic layer was removed, and the water evaporated under high vacuum. To the solid obtained were added 25 mL MeOH and the solids were
30 filtered off. Further purification was accomplished by gel chromatography Sephadex LH-20 using MeOH as eluent, to

give the peptide as a white solid.

¹H NMR (400 MHz, DMSO): δ 8.28 (bt, 1H), 7.74 (d, *J* = 8.2 Hz, 1H), 7.33-7.18 (m, 5H), 6.6 (bs, 1H), 6.24-6.14 (m, 1H), 5.02-4.90 (m, 2H), 4.39-4.17 (m, 5H), 3.85-3.49 (m, 4H), 2.90-2.82 (m, 2H), 2.77 (s, 6H), 2.08-1.22 (m, 10H).
¹³C NMR (300 MHz, DMSO): δ 172.4, 172.2, 158.9, 140.4, 129.1, 128.0, 127.7, 127.5, 63.1, 60.9, 55.8, 53.8, 47.7, 42.8, 36.7, 31.8, 30.1, 24.8, 23.3. LRMS (FAB): *M* = 490
calculated for C₂₄H₃₈O₅N₆. Found *m/z* = 491 (*M*+1).

Synthesis of control peptides (6) and (8)

BnNHD-ProBoc: To 500 mg (2.32 mmols) of BocProOH in 15 mL of DCM were added 187 μL (2.32 mmols) pyridine and 324 μL (3.48 mmols) cyanuric fluoride. The resulting solution was stirred under argon at r.t. for 1 h. After dilution with DCM to 150 mL, the organic layer was washed with 2 mL water. Solvent was removed, and to this crude material 253 μL (2.3 mmols) benzylamine and 800 μL (4.6 mmols) DIEA were added in 20 mL DCM. The solution was stirred at r.t. under argon for 1.5 h. Solvent was removed and after purification by silica gel column chromatography (DCM:acetone at 10:1), the peptide was obtained as 670 mg of white solid in 96% yield.

¹H NMR (400 MHz, CD₃OD): δ 7.27-7.37 (m, 4H), 7.20-7.29 (m, 1H), 4.43-4.46 (m, 1H), 4.27-4.31 (m, 1H), 4.13-4.22 (m, 1H), 3.50-3.58 (m, 1H), 3.38-3.46 (m, 3H), 2.18-2.31 (m, 1H), 1.81-2.02 (m, 3H), 1.47 (s, 3H), 1.33 (s, 6H). CIMS (NH₃): *M* = 305 calculated for C₁₇H₂₄O₃N₂. Found *m/z* = 306

(M+1).

BnNHD-Pro-L-Ser (tBut) Fmoc: To 304 mg (1 mmol) of BnNH-L-ProBoc were added 100 mL DCM:TFA at 4:1, and the solution
5 was stirred at r.t. for 1 h. The excess TFA was removed, and to a solution of this crude material in 25 mL DCM were added 400 mg (1.04 mmols) of the acid fluoride of FmocL-Ser(OtBut) and 2 eq. of DIEA. After stirring at r.t. for 45 min., the solvent was removed and the peptide was
10 purified by column chromatography (DCM:acetone at 9:1) to give 550 mg of a white foam in 97% yield.

CIMS (NH₃): M = 569 calculated for C₃₄H₃₉O₅N₃. Found m/z = 570 (M+1).

15 **BnNHD-Pro-L-Ser (tBut)NH₂:** Deprotection of the Fmoc group was achieved by stirring the substrate in a solution of DCM:Piperidine at 4:1 for 30 min. Solvent was removed, and purification of the crude by flash chromatography with DCM:MeOH at 8:1, gave 315 mg amine in 94% yield.

20 CIMS (NH₃): M = 347 calculated for C₁₉H₂₉O₃N₃. Found m/z = 348 (M+1).

BnNHD-Pro-L-Ser (tBut)NHCONMe₂: To the solution of amine in 10 mL DCM, DIEA and dimethylcarbamy chloride were added
25 in excess over a period of 5-7 h, until the TLC does not indicate the presence of the amine. Solvent was removed. Purification on a silica gel column with DCM:acetone at 1:1 gave 300 mg product in 80% yield.

30 ¹H NMR (400 MHz, CDCl₃): δ 7.98 (t, J = 6.1Hz, 1H), 7.28-7.15 (m, 5H), 5.15 (d, J = 6.1Hz, 1H), 4.72-4.25 (m, 4H),

4.06-3.55 (m, 4H), 2.59 (s, 6H), 2.35-1.92 (m, 4H), 1.17 (s, 9H). CIMS (NH₃): M = 418 calculated for C₂₂H₃₄O₄N₄. Found m/z = 419 (M+1).

5 **BnNHD-Pro-L-Ser (OH)NHCONMe₂ (6)**: Deprotection of the *t*-Butyl group was accomplished by stirring the peptide with a solution of DCM:TFA at 4:1 for 1 h at r.t.. Solvent was removed, and after purification by silica gel column chromatography (DCM:acetone:MeOH at 5:5:1), the product
10 was obtained as 110 mg of oil in 42% yield (87% yield based on recovered starting material). Longer reaction time or more TFA led to decomposition of starting material. Further purification was done by size-exclusion chromatography on Sephadex LH-20 with MeOH.
15 Recrystallization from DCM/hexane gave 100 mg of a white solid.

¹H NMR (400 MHz, DMSO): δ 8.24 (t, *J* = 6.1 Hz, 1H), 7.35-7.17 (m, 5H), 6.38 (d, *J* = 6.1 Hz, 1H), 4.72-4.25 (m, 4H),
20 4.06-3.55 (m, 4H), 2.59 (s, 6H), 2.35-1.92 (m, 4H), 1.17 (s, 9H). ¹³C NMR (300 MHz, CDCl₃): δ 171.11, 170.89, 157.37, 137.78, 128.04, 126.92, 126.75, 64.08, 59.94, 53.21, 47.11, 42.84, 35.66, 28.36, 24.27. HRMS (FAB): calculated for C₁₈H₂₆O₄N₄ (M+1) 363.2032, found 363.2019. IR
25 (polyethylene card): 3307, 2918, 2849, 1659, 1651, 1643, 1634, 1538, 1472, 1462, 1231, 1065.

BnNHL-LysNHAc (8): To 250 mg (0.74 mmols) of the benzylamide of L-Lys(δNHBoc) in 20 mL DCM were added 200
30 mL TEA, 200 mL Ac₂O and a catalytic amount of DMAP. After stirring the mixture at r.t. for 1 h, the solvent was removed and purification was performed on a silica gel

column with DCM:acetone at 1:1. After standard deprotection of Boc and removal of the excess TFA, 10 mL saturated NaHCO_3 were added and the free amine extracted several times with DCM. Further purification was achieved on a Sephadex LH-20 column with MeOH.

^1H NMR (300 MHz, DMSO): δ 8.55 (t, $J = 6.0$ Hz, 1H), 8.11 (d, $J = 8.0$ Hz, 1H), 7.32-7.18 (m, 5H), 4.28-4.12 (m, 3H), 2.52-2.42 (m, 2H), 1.82 (s, 3H), 1.66-1.16 (m, 6H). ^{13}C NMR (300 MHz, DMSO): δ 173.4, 172.5, 138.9, 128.5, 127.5, 127.2, 53.9, 43.0, 39.9, 31.6, 28.5, 23.0, 21.5. CIMS (CH_4): $M = 277$ calculated for $\text{C}_{15}\text{H}_{23}\text{O}_2\text{N}_3$. Found $m/z = 278$ ($M+1$).

To 0.5 mmols prolinol in 25 mL DCM were added 1.2 equivalents Boc protected aminoacid, 1.2 equivalents EDC and a catalytic amount of DMAP (Figure 12). After stirring the mixture at r.t. for 2 h, 100 mL of DCM were added and the solution was washed with 5 mL HCl 1 N. Solvent was removed and the product purified on silica gel column with DCM:acetone at 1:1 to give a colorless oil in yields higher than 90%.

SProC1NHBoc: ^1H NMR (400 MHz, CDCl_3): δ 5.48 (bt, 1H), 4.57 (d, $J = 6.1$ Hz, 1H), 4.28-4.18 (m, 1H), 4.02-3.78 (m, 2H), 3.74-3.40 (m, 4H), 2.12-1.78 (m, 3H), 1.67-1.62 (m, 1H), 1.47 (s, 9H). CIMS (CH_4): $M = 258$ calculated for $\text{C}_{12}\text{H}_{22}\text{O}_4\text{N}_2$. Found $m/z = 259$ ($M+1$).

SProC2NHBoc: ^1H NMR (400 MHz, CDCl_3): δ 5.28 (bt, 1H), 4.98 (bd, 1H), 4.28-4.18 (m, 1H), 3.74-3.54 (m, 2H), 3.51-3.42 (m, 4H), 2.54 (t, $J = 7.3$ Hz, 2H), 2.15-1.87 (m, 3H),

SProC3NH₂Boc: ¹H NMR (400 MHz, CDCl₃): δ 4.96 (bd, 1H), 4.75 (bt, 1H), 4.28-4.18 (m, 1H), 3.82-3.74 (m, 2H), 3.62-3.47 (m, 3H), 3.24-3.12 (m, 2H), 2.42-2.34 (m, 2H), 2.10-1.71 (m, 3H), 1.68-1.58 (m, 1H), 1.48 (s, 9H). ¹³C NMR (300 MHz, CDCl₃): δ 174.0, 156.7, 79.6, 67.4, 61.4, 48.5, 40.3, 32.4, 28.8, 28.6, 25.4, 24.8. CIMS (NH₃): M = 286 calculated for C₁₄H₂₆O₄N₂. Found m/z = 287 (M+1).

SProC4NHBoc: ^1H NMR (400 MHz, CDCl_3): δ 5.11 (bd, 1H), 4.65 (bt, 1H), 4.28-4.20 (m, 1H), 3.72-3.47 (m, 4H), 3.22-3.11 (m, 2H), 2.37 (t, 2H, $J = 7.3$ Hz), 2.13-1.81 (m, 3H), 1.74-1.52 (m, 4H), 1.47 (s, 9H). ^{13}C NMR (300 MHz, CDCl_3): δ 174.6, 156.5, 79.5, 68.0, 61.6, 48.5, 40.5, 34.8, 29.9, 28.8, 28.7, 24.8, 22.1. CIMS (NH_3): $M = 300$ calculated for $\text{C}_{15}\text{H}_{28}\text{O}_4\text{N}_2$. Found $m/z = 301$ ($M+1$).

20 **SProC5NHboc:** ^1H NMR (400 MHz, CDCl_3): δ 5.14 (d, J = 6.0 Hz, 1H), 4.54 (bt, 1H), 4.28-4.20 (m, 1H), 3.72-3.45 (m, 4H), 3.18-3.07 (m, 2H), 2.73 (t, J = 7.3 Hz, 2H), 2.12-1.72 (m, 3H), 1.74-1.42 (m, 7H), 1.47 (s, 9H). ^{13}C NMR (300 MHz, CDCl_3): δ 174.7, 156.5, 79.4, 67.7, 61.5, 48.5, 40.7, 35.3, 30.3, 28.8, 28.6, 26.9, 24.7. CIMS (NH_3): M = 314
25 calculated for $\text{C}_{16}\text{H}_{30}\text{O}_4\text{N}_2$. Found m/z = 315 ($M+1$).

SProC6NHBoc: ^1H NMR (400 MHz, CDCl_3): δ 5.28 (d, J = 6.0 Hz, 1H), 4.52 (bt, 1H), 4.29-4.21 (m, 1H), 3.74-3.42 (m,

4H), 3.18-3.07 (m, 1H), 2.72 (t, $J = 7.3$ Hz, 2H), 2.22-1.84 (m, 3H), 1.72-1.34 (m, 9H), 1.47 (s, 9H). ^{13}C NMR (300 MHz, CDCl_3): δ 174.7, 154.2, 68.2, 61.6, 48.5, 35.4, 30.3, 29.4, 28.8, 28.7, 26.9, 25.0, 24.8. CIMS (NH_3): $M = 228$
5 calculated for $\text{C}_{17}\text{H}_{32}\text{O}_4\text{N}_2$. Found $m/z = 229$ ($M+1$).

Deprotection of Boc was achieved with TFA:DCM at 1:3 in 30 min. After the excess TFA was removed on high vacuum, the salt was dissolved in 1 mL water and added to a small
10 Dowex 50Wx4-400 ion exchange column, and eluted with a solution of NH_4OH 1 M. Further purification was achieved using a C18 cartridge (Waters), eluting with water. The amine was obtained in 80% overall, as a colorless oil.

15 **SProC1**: ^1H NMR (400 MHz, DMSO): δ 3.96-3.78 (m, 1H), 3.53-3.15 (m, 6H), 1.94-1.72 (m, 4H). ^{13}C NMR (300 MHz, DMSO): δ 172.4, 63.3, 61.9, 59.6, 58.6, 46.2, 44.5, 28.7, 27.3, 24.2, 21.9. CIMS (NH_3): $M = 158$ calculated for $\text{C}_7\text{H}_{14}\text{O}_2\text{N}_2$. Found $m/z = 159$ ($M+1$).

20

SProC2: ^1H NMR (400 MHz, DMSO): δ 4.01-3.85 (m, 1H), 3.52-3.08 (m, 6H), 2.52-2.34 (m, 2H), 1.98-1.77 (m, 4H). ^{13}C NMR (300 MHz, DMSO): δ 171.2, 63.3, 62.1, 59.4, 59.3, 47.7, 46.1, 28.6, 27.6, 24.3, 22.2. CIMS (NH_3): $M = 172$
25 calculated for $\text{C}_8\text{H}_{16}\text{O}_2\text{N}_2$. Found $m/z = 173$ ($M+1$).

SProC3: ^1H NMR (400 MHz, DMSO): δ 3.95-3.87 (m, 1H), 3.54-3.20 (m, 5H), 2.94 (bt, 2H), 2.94-2.72 (m, 2H) 1.96-1.52 (m, 6H). ^{13}C NMR (300 MHz, DMSO): δ 171.9, 63.3, 62.1,
30 59.3, 59.1, 47.6, 46.1, 32.3, 31.7, 28.6, 27.6, 24.3,

22.2. CIMS (NH_3): $M = 186$ calculated for $\text{C}_9\text{H}_{18}\text{O}_2\text{N}_2$. Found $m/z = 187$ ($M+1$).

SProC4: ^1H NMR (400 MHz, DMSO): δ 3.95-3.85 (m, 1H), 3.49-
5 3.09 (m, 4H), 2.94-2.48 (m, 2H) 2.37-2.12 (m, 2H), 1.96-
1.34 (m, 8H). ^{13}C NMR (300 MHz, DMSO): δ 171.9, 63.3, 62.1,
59.4, 59.3, 47.6, 46.1, 42.1, 34.6, 33.9, 32.3, 28.6,
27.5, 24.3, 22.9, 22.6, 22.1, 21.6. CIMS (NH_3): $M = 200$
calculated for $\text{C}_{10}\text{H}_{20}\text{O}_2\text{N}_2$. Found $m/z = 201$ ($M+1$).

10

SProC5: ^1H NMR (400 MHz, DMSO): δ 3.88-3.74 (m, 1H), 3.52-
3.22 (m, 4H), 2.91-2.52 (m, 2H), 2.39-2.19 (m, 2H) 1.95-
1.74 (m, 4H), 1.54-1.22 (m, 6H). ^{13}C NMR (300 MHz, DMSO): δ
171.9, 63.4, 62.2, 59.4, 59.3, 47.6, 46.1, 34.8, 34.1,
15 28.6, 27.5, 26.9, 25.6, 24.9, 24.3, 22.1. HRMS (FAB):
calculated for $\text{C}_{11}\text{H}_{22}\text{O}_2\text{N}_2$ 214.3079. Found 214.1619.

RProC5: ^{13}C NMR (300 MHz, DMSO): δ 171.8, 63.3, 62.1, 59.4,
59.3, 47.6, 46.1, 34.6, 33.9, 29.0, 28.6, 27.5, 26.5,
20 25.3, 24.7, 24.3, 22.1. CIMS (NH_3): $M = 214$ calculated for
 $\text{C}_{11}\text{H}_{22}\text{O}_2\text{N}_2$. Found $m/z = 215$ ($M+1$).

SProC6: ^1H NMR (400 MHz, DMSO): δ 4.01-3.70 (m, 1H), 3.52-
3.18 (m, 4H), 2.93-2.48 (m, 2H), 2.39-2.14 (m, 2H) 1.95-
25 1.68 (m, 4H), 1.54-1.18 (m, 8H). ^{13}C NMR (300 MHz, DMSO): δ
172.0, 63.3, 62.1, 59.4, 47.6, 46.1, 34.8, 34.1, 29.5,
28.6, 27.5, 27.1, 25.8, 25.2, 24.3, 22.1. CIMS (NH_3): $M =$
228 calculated for $\text{C}_{12}\text{H}_{24}\text{O}_2\text{N}_2$. Found $m/z = 229$ ($M+1$).

Preparation of in vivo study samples

For the in vivo experiments, deprotection of the S,RProCnNHBoc derivatives was performed by stirring for 1 h in 3 M HCl in EtOAc. Solvent was removed and the oils were extensively dried on high vacuum. 100 mM stock solutions of each derivative were made in sterile PBS pH 7.4 and the pH of the resulting solutions was adjusted with concentrated NaOH to 7.

10

Figure 13. Step (a). To 1 mL (9.9 mmols) diamine in 100 mL DCM were added 2.45 g (9.9 mmols) BocON and 1.4 mL TEA, and the solution was stirred at r.t. overnight. The product was purified on silica gel column with DCM:MeOH at 1:1 (5% TEA) to give 1.8 g of white solid in 96% yield.

¹H NMR (400 MHz, CDCl₃): δ 4.68 (bt, 1H), 3.18 (bq, 2H), 2.72 (t, *J* = 6.7 Hz, 2H), 1.60–1.50 (m, 2H), 1.52 (s, 9H), 1.31–1.21 (m, 2H).

20

Figure 13. Step (b). To 83 mg (0.5 mmols) of L-ProOMe·HCl were added 100 μ L TEA followed by 1 mL solution 2 M of phosgene in toluene, and the mixture was stirred at r.t. for 1 h. After a careful removal of excess phosgene, the product was dissolved in 5 mL DCM and added to a solution of 140 μ L (1.3 mmols) pyridine and 94 mg (0.5 mmols) amine in 15 mL DCM. The solution was stirred at r.t. for 2 h. Solvent removal, followed by purification on silica gel column with DCM:acetone at 1:1 gave 150 mg of product as a colorless oil, in 90% yield.

¹H NMR (400 MHz, CDCl₃): δ 4.67-4.58 (m, 1H), 4.57-4.51 (m, 1H), 4.47-4.41 (m, 1H), 3.74 (s, 3H), 3.52-3.22 (m, 4H), 3.19-3.08 (m, 2H), 2.17-1.97 (m, 4H), 1.60-1.50 (m, 4H), 1.48 (s, 9H). CIMS (NH₃): M = 343 calculated for C₁₆H₂₉O₅N₃.

5 Found m/z = 344 (M+1).

Figure 13. Step (c). To the methyl ester were added 166 mg (10 eq.) NaBH₄ in 15 mL MeOH and the solution was stirred at r.t. for 20 min and then refluxed for 2 h. Purification on silica gel column with DCM:acetone at 1:1 gave the product quantitatively as a colorless oil.

¹H NMR (400 MHz, CDCl₃): δ 5.18 (bd, 1H), 4.86 (bd, 1H), 4.65 (bt, 1H), 4.17-4.05 (m, 1H), 3.72-3.52 (m, 2H), 3.40-3.11 (m, 6H), 2.11-1.87 (m, 2H), 1.62-1.57 (m, 4H), 1.48 (s, 9H). ¹³C NMR (300 MHz, CDCl₃): δ 159.6, 156.7, 79.5, 68.0, 60.6, 47.2, 40.7, 40.6, 29.0, 28.8, 27.8, 27.7, 24.5. CIMS (NH₃): M = 315 calculated for C₁₅H₂₉O₄N₃. Found m/z = 316 (M+1).

20

Figure 13. Step (d). Deprotection of the Boc group and work up of the resulting amine, were completed as described for ProCn.

¹H NMR (400 MHz, DMSO): δ 6.37 (bt, 1H), 3.71-3.63 (m, 1H), 3.44-3.18 (m, 4H), 3.12-2.90 (m, 2H), 2.73 (bt, 2H), 1.88-1.68 (m, 4H), 1.55-1.35 (m, 2H). ¹³C NMR (300 MHz, DMSO): δ 158.4, 64.5, 59.5, 47.0, 28.4, 27.9, 24.1. CIMS (CH₄): M = 215 calculated for C₁₀H₂₁O₂N₃. Found m/z = 216 (M+1).

30

In vitro hydrolysis of D-Ala-D-Lac

The ability of peptide **4a** in cleaving the substrate **5** was assessed in aqueous phosphate buffer pH 7 at 37°C. Using HPLC and monitoring the *p*-NO₂-phenyl derivative by UV at 275 nm, we could easily follow the disappearance of D-Ala-D-Lac derivative **5** and the formation of the hydrolyzed product **4**. No significant effect on the rate of hydrolysis over buffer was observed using the control sequences **6**, **7**, **8** alone or **6** and **8** combined (Figure 14). A 20% hydrolysis of D-Ala-D-Lac in the presence of **4a** was observed after 24 h.

The prolinol derivatives were additionally, tested for their ability to cleave **5** in aqueous phosphate buffer pH 7.0 at 37°C. A 50% D-Ala-D-Lac hydrolysis was observed after 24 h implying that the **SProC5** derivative is twice as active as the initial peptide **4a**. Activity declines in the SProCn series with the decrease of the chain length from 5 carbons to 1 carbon. This result can be explained not only by a decrease in efficiency of the terminal amino in reaching to the carboxylate and ester of D-Ala-D-Lac with the shortening of the chain, but also by the decrease in nucleophilicity of the hydroxyl. A 6 carbon chain is also less active. The lower activity of SProC6 is probably due to the higher flexibility of the carbon chain that does not render the amino group available for the reaction.

To confirm these speculations we compared the chemical shifts of the OH and NH in the H¹ NMR spectra of the NHBoc protected SProCn series (Table 7). The study shows that there is a competition between the amino group and the hydroxyl for hydrogen bonding to the amide. With the

decrease of the chain length, the probability of the OH being hydrogen bonded decreases substantially, fact reflected in the chemical shifts of the OH and NH with the change in the length of the carbon chain.

5

Table 7. Shifts in the NMR position of OH and NHBoc (Boc-protected small molecule series) with the modification of the carbon chain length.

Boc- derivative	OH position (ppm)	NH position (ppm)
SProC1	4.57	5.48
SProC2	4.98	5.28
SProC3	4.96	4.75
SProC4	5.11	4.65
SProC5	5.14	4.54
SProC6	5.28	4.52
SProUC4	4.86	4.65

10

The table also explains the low activity of SProUC4, derivative in which the amide is replaced by urea. The urea is a better acceptor than the amide and should increase the reactivity of the hydroxyl. However, it is possible that structural constraints imposed by the urea play an important role and do not allow for proper orientation for H-bonding.

15

Mechanistic studies

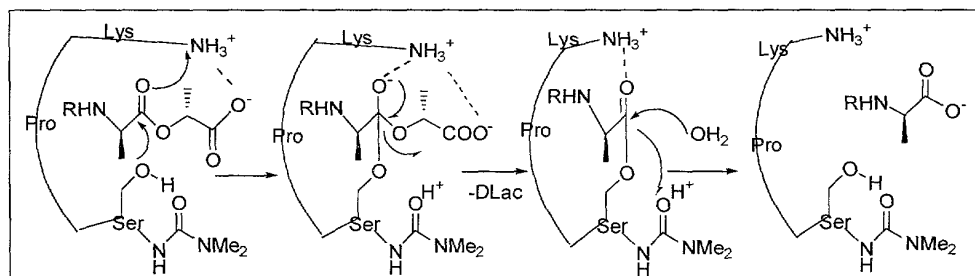
20

To get insights on the mechanism of this reaction, the cleavage of **1** by **4a** was studied in THF-5% water. Stock

solutions of 2 mM concentration of **1** in THF and 49 mM of **4a** (lyophilized from PIPES buffer pH 7.0) in water were prepared. In three ampoules were added 40 μ L solution of **1**, 7 μ L of **4a** and the volume was adjusted to 160 μ L with THF. For background measurements, in another three ampoules 7 μ L water were added instead of **4a** to the solution of **1**. All six ampoules were sealed under an argon stream and placed in an oil bath heated at 60°C. For each measurement one vial was opened and 5 μ L were taken, diluted with 5 μ L THF and 2 μ L of this solution were injected in the HPLC.

The reaction proceeds with the formation of a transesterification product **3**, which then is cleaved by water (Scheme 3).

Scheme 3



The reaction could be monitored by HPLC at 485 nm, and the separation of the three components was easily performed on an analytical reverse phase column using a gradient of acetonitrile : water. Isolation of the intermediate **3** proved to be however, more difficult. Application of the assay mixture to a size exclusion column (Sephadex LH-20 with DMF) gave a fraction enriched in **3**, and this was used for a COSY-¹H NMR analysis (Figure 17). Comparison of the

NMR spectra of 4a, 3 and 9 (Figure 16) confirmed the identity of the intermediate 3. Mass spectrum analysis (MS) was used to additionally establish the identity of the three peaks seen in the HPLC chromatogram (Figure 15).

5 The above description is for the purposes of teaching the person of ordinary skill in the art how to practice the invention, and is not intended to detail all those obvious modifications and variations of it, which will become apparent to the skilled worker upon reading the
10 description. It is intended, however, that all such obvious modifications and variations be included within the scope of the present invention, which is defined by the following claims.

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